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EFFECT OF DUAL-SACCHARIDES ON MICROBIAL CELLULOSE (MC) PRODUCTION BY *Pseudomonas aeruginosa*

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ABSTRACT

Microbial cellulose (MC) is an alternative source of cellulose that can be produced by different types of microorganisms such as *Pseudomonas*, *Komagataeibacter*, *Sarcina* and *Azotobacter* and they are commonly used in biomedical applications. However, the main issue in MC production is low yield. To enhance MC production, different combinations of the dual-saccharides, such as 25 g/L glucose + 25 g/L galactose, 25 g/L glucose + 25 g/L fructose, 25 g/L glucose + 25 g/L maltose and 25 g/L glucose + 25 g/L lactose were added into culture medium. The fermentation was carried out at 30°C for 5 days in incubator shaker. The combination of glucose and galactose (1:1) gave a higher concentration of MC with 1.23 g/L ± 0.15. However, it was lower than glucose (control) (1.8 g/L ± 0.63). The glucose + galactose concentrations varied between 10 g/L, 30 g/L, 50 g/L and 70 g/L for the MC production. Therefore, the concentration of 50 g/L is the best for the the MC production. Increasing the concentration of glucose + galactose beyond 50 g/L decreased MC production.

Key words: Microbial cellulose, fermentation, *Pseudomonas aeruginosa*, dual-saccharide

INTRODUCTION

Microbial cellulose (MC) is an extracellular polysaccharide mostly produced by acetic acid bacteria from the genus *Acetobacter* (Chawla *et al.,* 2009; Campano *et al.,* 2016). MC is considered as a high pure cellulose because it does not contain the contaminants of hemicelluloses and lignin (Raghunathan, 2013). MC has many of the unique characteristics such as high purity, high mechanical tensile strength, higher degree of polymerization, high crystallinity index and high absorption of water (Chawla *et al.,* 2009). MC becomes a promising and highly functional biomaterial in food and textile industries, including biomedical agent (Wu *et al.,* 2014). MC is more preferred compared to plant cellulose as the growth of bacteria can be controlled under the fixed conditions and the yields of MC can be increased by utilizing the different type carbon sources such as glucose, galactose, fructose, maltose, lactose, sucrose and mannitol (Fadilah, 2010).

In this study, *Pseudomonas aeruginosa* is chosen for its use in producing MC because it has a good potential to produce high yields of MC under the suitable culture conditions (Aydin, 2009; Stasiak & Blazejak, 2009). The carbon source is the main factor that affects the MC production. Generally, the culture media used for MC production requires a carbon and nitrogen source and salts to perpetuate the pH (Saichana *et al.,* 2015) during fermentation process. Numerous attempts have been made in enhancing MC production by utilizing different carbon sources such as sucrose, glucose, fructose, mannitol and fructose (El-Saied *et al.,* 2004; Molina-Ramirez *et al.,* 2017). Based on the research by Hungund *et al*. (2013), the combination of two carbon sources can enhance the MC production by *Gluconacetobacter persimmonis*. Nevertheless, the effect of dual-saccharides on the MC production by *Pseudomonas aeruginosa* is largely unknown.

Hence, this study aims to investigate the effect of combinations of glucose with other saccharides such as galactose, fructose, maltose and lactose on the MC production and to determine MC concentration by varying the best combination's concentration. Since there are less studies about the dual-saccharides on the MC production, therefore this study about of effect of dual-saccharides on MC production might be considered as the preliminary

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research to enhance the yields of MC. By utilizing dual-saccharides in the fermentation culture medium, the MC could be increased.

MATERIALS AND METHODS

Preparation of *P. aeruginosa* Culture

Microorganism

P. aeruginosa culture was provided by Microbiology Laboratory, School of Fundamental Science, UMT.

Culture media (Adnan, 2015)

Fifty g of D-glucose, 5 g ammonium sulphate, 3 g potassium hydrogen orthophosphate, 5 g yeast extract and 0.05 g magnesium sulphate were added into the 250-mL shake flasks. Media pH was adjusted to 6.8 with 1M NaOH, and then was autoclaved at 121°C for 15 minutes.

Inoculum media

A loop full of bacteria was gently scraped from the surface of solid agar and transferred into 80 mL of 50 g/L glucose growth media together with other components described previously in a 250-mL conical flask. The flask was plugged with cotton wool and was incubated in incubator shaker (Innova 44) with agitated speed of 150 rpm at 30°C for 5 days until MC pellicles appeared.

Determination of MC production by *P. aeruginosa* using different combination of Dual-Saccharides

To investigate the effect of dual-saccharides, combinations of sugars/saccharides were added to media with a pH of 6.8. Different combinations of sugar such as 25 g/L glucose + 25 g/L galactose, 25 g/L glucose + 25 g/L fructose, 25 g/L glucose + 25g/L maltose and 25 g/l glucose + 25 g/L lactose were added into the broth, with the composition of 5 g/L of yeast extract, 5 g/L ammonium sulphate $(NH_4)_2SO_4$, 3 g/L of potassium dihydrogen phosphate (KH_2PO_4), 0.05 g/L of magnesium sulphate ($MgSO_4$). All fermentation trials were done at an initial pH of 6.8, 30°C, 150 rpm for 5 days. For every 24 hours, the broth from shake flasks was harvested aseptically and then homogenized (Wiggen) at 100 rpm for 5 minutes. The samples were used to determine MC concentration through MC purification and determination steps. The best sugar/saccharide combination was used in the further experiments.

MC purification and determination (Cheng *et al,* 2009)

The washed pellets from samples were treated with 1M NaOH at 90°C for 30 minutes to dissolve cells. The MC obtained were centrifuged (Eppendorf 5804R) at 4000 rpm for 20 minutes, washed with distilled water, and oven-dried at 80°C for 24 hours, and were weighed using analytical balance (Mettler Toledo pb303-L).

Production of MC by varying the concentration of best combination of Dual-Saccharides

The selected combination of dual-saccharides was used to optimize the MC production by varying the concentration. The varied concentrations used were 10 g/L, 30 g/L, 50 g/L and 70 g/L. According to a study by Adnan *et al.* (2015), the concentration beyond 40 g/L of carbon sources would able to reduce MC production. Thus, these concentrations had been chosen to determine the bet concentration to the MC with the best combination of dual-saccharides. Fermentation was done at 30°C with 150 rpm for five days. The samples, which taken every 24 hours were used for MC purification and determination. The MC concentration was determined in triplicates and was reported as average in form of a graph.

RESULTS

Effect of Dual-Saccharides on MC production by *P. aeruginosa*

There were four combinations of dual-saccharides, which were utilized for MC production by *P. aeruginosa,* namely glucose + galactose, glucose + fructose, glucose + maltose, glucose + lactose and glucose as the control. Based on Figure 1, the average MC concentration produced by utilizing 50 g/L glucose as carbon source was 1.8 ± 0.63 g/L. The graph depicted a gradual increment trend from day 0 to day 5. It was increased gradually from day 1 (0.3 ± 0.06 g/L) until day 5 (1.8 ± 0.63 g/L). For the combination of glucose and galactose, the graph showed a steady increment trend from day 0 to day 5. This combination gave 0.33 ± 0.06 g/L of MC concentration on first day and gave 1.23 ± 0.15 g/L of MC concentration on the fifth day. In addition, the combination of glucose and fructose, also showed an increment trend from day 0 to day 5. However, this graph showed that the MC production was increased slowly from 0 g/L of MC concentration on day 0 to 0.2 ± 0.1 g/L of MC concentration on day 2 and then it was increased steadily from second day to fifth day. At the end of the fermentation, 0.93 ± 0.35 g/L of MC concentration was produced.

For glucose and maltose combination, supplementing the culture media with 25 g/L glucose and 25 g/L maltose resulted in 1.1 ± 0.17 g/L of MC concentration. It was increased

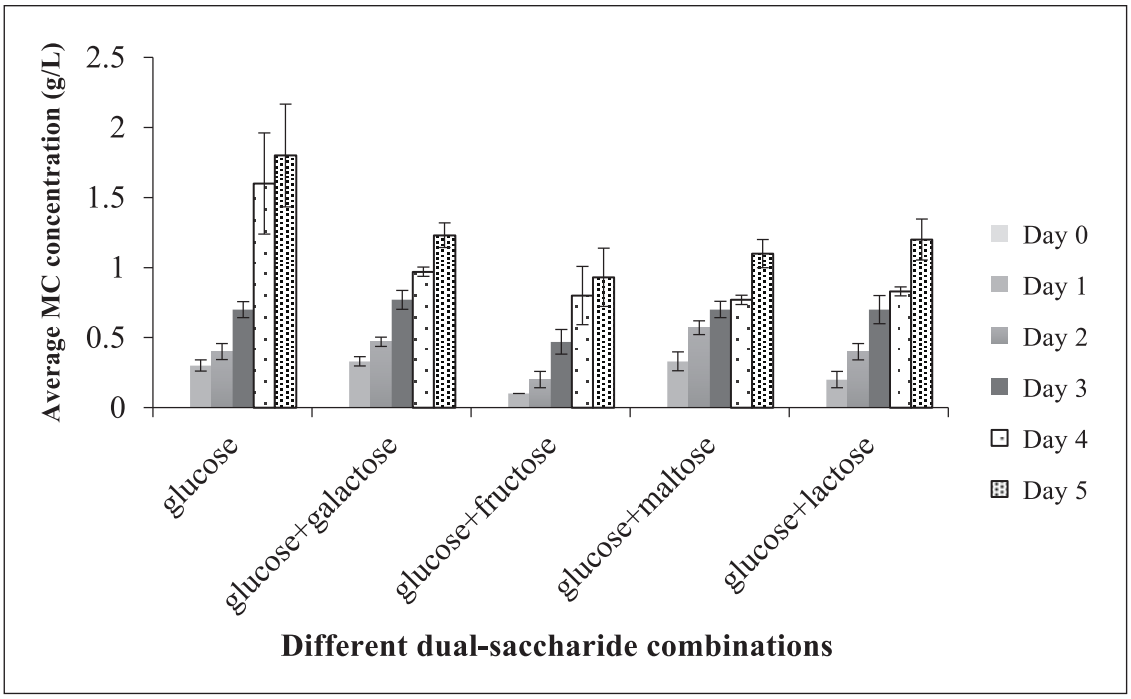

Fig. 1. Effect of various carbon sources combinations on MC concentration by *Pseudomonas aeruginosa*.

significantly from day 0 (0 g/L) to day 1 (0.33 ± 0.09 g/L), however, a slight increment trend was obtained from day 1 to day 5 in this fermentation trial. Meanwhile, glucose and lactose combination showed a gradual increment when fermenting *P. aeruginosa* in glucose and lactose media, which yielded 1.2 ± 0.17 g/L MC. There was an initial increase of MC concentration from day 0 (0 g/L) to day 3 (0.7 ± 0.17 g/L) and then the increment of MC concentration slowly occurred until day 4 (0.83 ± 0.08 g/L). The best combination of dual-saccharides for the MC production is a combination between glucose and galactose, followed by glucose and lactose, glucose and maltose, glucose and fructose, which gave the amount of MC concentration of 1.23 ± 0.15 g/L, 1.2 ± 0.25 g/L, 1.1 ± 0.17 g/L and 0.93 ± 0.35g/L, respectively. From Figure 1, it could be concluded that the best carbon source for MC production was glucose, whereas the best combination of dual-saccharides was the combination of glucose and galactose.

Effects of Dual-Saccharide combinations on MC volumetric productivity

Table 1 shows the MC volumetric productivity from each dual-saccharide by *P. aeruginosa*. The highest MC productivity by *P. aeruginosa* was obtained in glucose media, which gave 0.012 ± 0.004 g/L/day. Glucose and galactose media produced 0.007 ± 0.001 g/L/day of MC, while glucose and lactose yielded 0.006 ± 0.001 g/L/day of MC. Adding glucose and maltose gave 0.003 g/L/day of MC, followed by glucose and fructose (0.005 ± 0.004 g/L/day). Hence, glucose + galactose was the best dual-saccharide combination to increase volumetric productivity as well.

Effect of varying glucose and galactose concentration on MC production by *P. aeruginosa*

In further experiments, four different concentrations such as 10g /L, 30 g/L, 50 g/L and 70 g/L were used to produce the MC as shown in Figure 2. Based on Figure 2, in 10 g/L of glucose and galactose combination, a total of 5 g/L glucose and 5 g/L galactose were supplemented to the culture media and this concentration produced 1.13 ± 0.15 g/L MC at the end of fermentation. A slight increment was observed from day 2 to day 3, while a drastic increment occurred from day 1 to day 2.

For the 30 g/L of glucose and galactose combination, a total of 15 g/L glucose and 15 g/L galactose was supplemented to the culture media.

Table 1. Effect of different combination of dual-saccharides on MC volumetric productivity by *Pseudomonas aeruginosa*

Parameters	Volumetric productivity (g/L/day)
Glucose (control)	0.012 ± 0.004
Glucose+Galactose	0.007 ± 0.001
Glucose+Fructose	0.005 ± 0.004
Glucose+Maltose	0.003 ± 0.04
Glucose+Lactose	0.006 ± 0.001

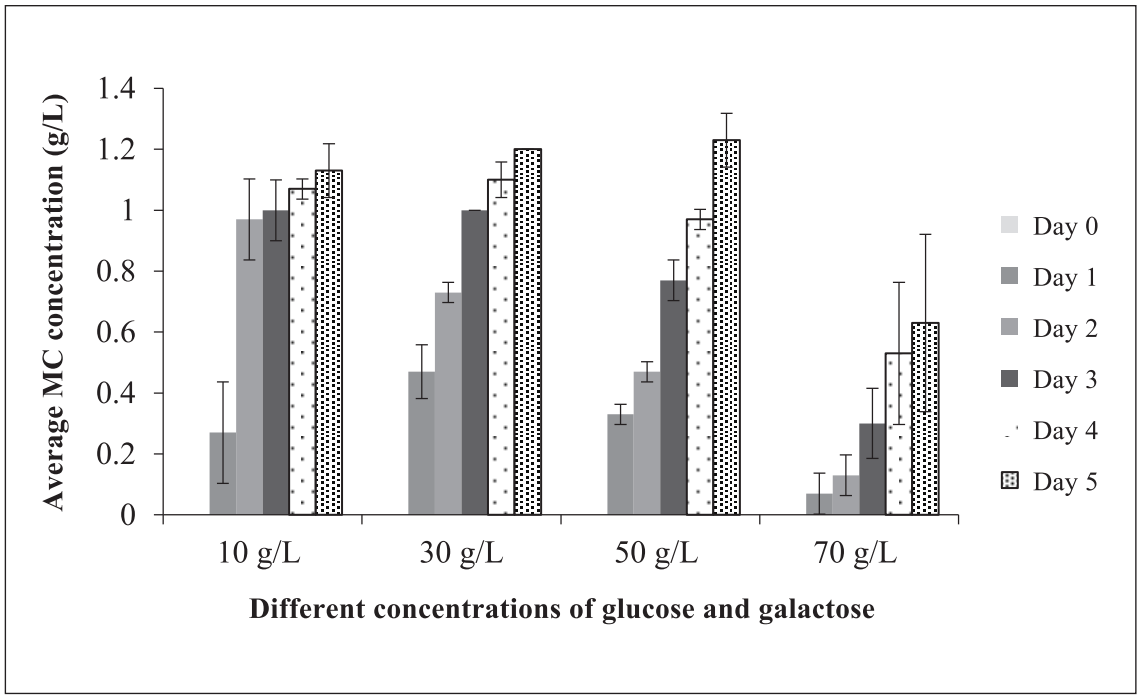

Fig. 2. Effect of different concentration of glucose and galactose combination on MC production by *Pseudomonas aeruginosa*.

At the end of fermentation, 1.2 ± 0.00 g/L of MC concentration produced. An increment was observed from day 0 to day 1, while a steady increment occurred from day 1 until day 5. This showed the higher MC concentration at end of fermentation compared to 10 g/L of glucose and galactose combination.

Figure 2, about 50 g/L of glucose and galactose, depicted the final concentration resulting from the utilization of 25 g/L glucose and 25 g/L galactose. A gradual increment trend was obtained from day 1 to day 5 in this fermentation trial. There were approximately 1.23 ± 0.15 g/L of MC concentration which was resulted at the end of this fermentation, whereas based on the graph of 70 g/L glucose and galactose media, the supplementing of 35 g/L glucose and 35 g/L galactose on MC production by *P. aeruginosa* gave 0.63 ± 0.5 g/L of MC concentration at the end of fermentation. The graph revealed an increment trend, however, the final MC concentration was the lowest compared to 10 g/L, 30 g/L and 50g/L.

Therefore, it indicated a comparison between the varying concentrations of glucose and galactose on MC concentration by *P. aeruginosa*. A gradual increment trend was obtained from 10 g/L to 50 g/L, however, a significant decrement was occurred from 50 g/L to 70 g/L. The highest final MC concentration was obtained from utilization of 50 /L glucose and galactose (1.23 ± 0.15 g/L), whereas the lowest final MC concentration was resulted from utilization of 70 g/L glucose and galactose (0.63 ± 0.5 g/L).

Volumetric productivity of MC by varying concentration of glucose and galactose combination by *P. aeruginosa*

Table 2 showed the MC volumetric productivity of different concentration of glucose + galactose. Ten, fifty and thirty g/L of glucose and galactose media gave the highest value of volumetric productivity, which was 0.008 ± 0.001 g/L/day, whereas 70 g/L glucose and galactose revealed the lowest volumetric productivity with 0.004 ± 0.003

Table 2. Effect of different concentration of glucose + galactose on MC volumetric productivity by *Pseudomonas aeruginosa*.

Different concentration of Glucose + galactose (g/L)	Volumetric productivity (g/L/day)
10	0.008 ± 0.001
30	0.008 ± 0.000
50	0.008 ± 0.001
70	0.004 ± 0.003

FORMULA:
Rate of MC production (Volumetric productivity)
= MC concentration (g/L) / (final volume × time)

Time = duration of fermentation (5 days)
Final volume = final volume of fermentation remained after 5 days)

g/L/day. Hence, increasing the concentration of saccharides beyond 50 g/L decreased the MC volumetric productivity.

DISCUSSION

Effect of Dual-Saccharides on MC production by *P. aeruginosa*

Based on Figure 1, the graph indicated increasing trend from day 0 to day 5 under shake flask culture. This is due to the amount of MC concentration is relative to the total sugar consumed and it is reduced with the increase of the initial concentration of sugar used (Masaoka *et al.,* 1993; Adnan, 2015). Thus, the longer the period of cultivation, the higher the bacterial growth, the higher the total amount sugar consumed, the higher the MC production by *P. aeruginosa* (Coban & Biyik, 2011). That is the reason that all the dual-saccharides combinations depicted the same trends of MC concentrations starting from day 0 to day 5.

For day 0, all graphs showed no MC produced by *P. aeruginosa* as it is assumed that cells are adapting to the culture conditions and unable to synthesize MC within the short period of time by utilizing the carbon sources provided. From day 0 to day 1, the MC concentration of glucose and galactose combination was different from other combinations because different carbon sources or different combination of dual-saccharides would have different time of initiation of MC synthesis (Chawla *et al.,* 2009). Besides, all graphs indicated that there were very small amount of MC produced from day 0 to day 2. This condition could be explained based on the growth phase of the *P. aeruginosa*. From day 0 to day 2, the bacteria undergoes a lag phase, during which they are adapting themselves to the culture medium conditions. In this phase, they are maturing and still unable to multiply to synthesize enzymes or RNA, hence, they cannot fully utilize the presence of carbon source to produce end products (Abbas *et al*., 2013; Chance & Mawhinney, 2017). A study by Pla *et al.* (2015) stated that the production of MC by bacteria is influenced by the physiological state of the bacterial cells and it depends on the culture media surrounding. Also, a stress situation presence can increase the cell lag phase durations. During third to fifth day, it is presumed that *P. aeruginosa* enter another growth phase, which known as log phase. During this phase, the bacteria have already adapted to the culture media conditions and they are able to multiply and start to synthesize RNA and enzymes such as cellulose synthase to oxidize different source of carbon source (Ross *et al.,* 1991; Adnan, 2015). Hence, the cell growth increases and the oxygen consumption is high which is directly proportional to the MC production as *P. aeruginosa* is an aerobic microorganism (Rashid, 2008; Gullo *et al.,* 2014).

Based on Figure 1, glucose gave the highest MC concentration by *P. aeruginosa* because glucose is a master carbon source and also acts as a precursor for the MC synthesis (Santos *et al.,* 2013; Farag *et al.,* 2016). Based on a study conducted by Kesk and Sameshima (2005), culture media containing glucose as a carbon source is the most suitable for the bacteria growth and they discovered that *P. aeruginosa* is capable to utilize glucose and going through polymerisation process to produce MC. Besides, a research carried out by Raghunathan (2013) also reported that glucose is able to synthesize highest MC in shake flasks culture compared to sucrose, lactose and maltose by *Acetobacter xylinus*. In 2002, Son *et al.* (2002) found that when the glucose concentration maintained within the range of 1% – 1.5%, MC production will be enhanced, however, if glucose concentration is beyond to 2%, it is too concentrated and decreased the MC concentration (Keskh & Sameshima, 2005).

The core issue of using glucose as the carbon source is the formation of gluconic acid in the culture medium, which lowers the pH of the medium and affects the yield of MC produced (Tsouko *et al.,* 2015). However, from the result obtained, the combination of dual-saccharides gave the lower MC concentration than control. This result is in agreement with the research done by Embuscado *et al.* (1994), which claimed the presence of glucose in combination with other saccharides in culture media can lower the MC concentration produced by *P. aeruginosa.* Therefore, it can be concluded that the combination of glucose and galactose can act as alternative carbon source to produce MC but it would not recommended in industrial scale since it did not enhance MC concentration compared to control.

When comparing glucose and galactose combination with other combinations, glucose and galactose media gave the higher MC concentration as it may be due to the presence of invertase inside *P. aeruginosa*, which can interconvert between UDP-galactose and UDP-glucose with the presence of UDP- Gal epimerase (Koivistoinen, 2013; Suzuki *et al.,* 2017). UDP- glucose acts as a precursor for the synthesis of MC and this is catalyzed by cellulose synthase to produce MC microfibril (Fadilah, 2010; Chen, 2014; Dayal, 2014; Adnan, 2015). Due to this feature, glucose and galactose gave higher MC concentration compared to others combinations as shown in Figure 1. However, when compared to a previous study by Hungund *et al.* (2013), the combination of glucose and galactose gave the

lowest MC concentration, which is contradict with the result obtained in this study.

Although a study by Hungund *et al.* (2013) claimed that the combination between glucose and fructose gave highest MC yields, it did not depict the same effect in this study. Low MC concentration could be due to the presence of fructose in the culture medium, as *P. aeruginosa* weakly respond to the presence of fructose (Kazim, 2015). In addition, a lacking an enzyme in their cells to convert the UDP-fructose to UDP-glucose for MC synthesis could be a reason that contribute to the above situation (Fadilah, 2010). Therefore, *P. aeruginosa* is incapable to fully utilize the combination of glucose and fructose to form large amount of MC as efficient as the combination between glucose and galactose.

Meanwhile, glucose and maltose media combination and glucose and lactose media combination produced MC concentration such as 1.1 ± 0.17 g/L and 1.2 g/L ± 0.25, respectively. This proved that *P. aeruginosa* is not efficient to utilize the disaccharides to form MC. From a previous study by Farag *et al.* (2016), the author claimed that lactose is not the preferred carbon source used to produce MC as they gave low MC concentration by *Achromobacter* species. Adnan (2015) also stated that *G. xylinus* grow weakly on the agar and media containing lactose. From Table 1, the control gave the highest volumetric productivity of MC because it produced highest MC concentration compared to others. By comparing glucose and galactose with other combination of dual-saccharides, it had higher MC volumetric productivity because the higher the concentration of MC produced, the higher the volumetric productivity of MC as they are incubated in the same volume of media, environmental factors and period of cultivation.

Effect of Dual-Saccharide combinations on MC volumetric productivity

Based on Table 1, the control gave the highest volumetric productivity of MC because it produced highest MC concentration compared to others. By comparing glucose and galactose with other combination of dual-saccharides, it had higher MC volumetric productivity because the higher the concentration of MC produced, the higher the volumetric productivity of MC as they are incubated at the same volume of media, environmental factors and period of cultivation.

Effect of different concentration of glucose and galactose combination on MC production under shake flask condition

The selected media combination was used in further experiments. Figure 2 showed the amount of MC concentration produced at different concentration of glucose and galactose combination. Based on the Figure 2, it shows a small amount of MC concentration was produced on day 1 as the inoculated bacteria were slowly responding to the media to produce MC as mentioned before. The drastic increment of MC concentration produced from day 1 to day 2 as there were sufficient nutrient and carbon source for the cell growth and MC production at early stage of growth phase (Pla *et al.,* 2015). Meanwhile, a slight increment of MC concentration from day 2 to day 5 was observed because there was limited supply of carbon sources as energy source for production MC. Although the adequate supply of other nutrients, the MC concentration could not increase significantly.

Furthermore, the MC concentration produced could be affected by the oxygen transfer rate (Cheng, 2010). The limited supply of the incubator shaker in the laboratory caused many students required to share the incubator shaker and influenced the availability of oxygen flow inside it. Hence, the unstable flow of oxygen in the culture media, the growth of bacteria was interrupted and caused them incapable to synthesize MC with a steady increment trend. Based on graph of 30 g/L glucose and galactose combination as shown in Figure 2, a dramatic increase of MC concentration from day 0 to day 1 because the higher the concentration of media, the higher the carbon source supply, therefore, the higher the utilization of carbon source to produce MC. A slight increment trend from day 1 to day 5 could be due to the oxygen transfer rate same as explanation mentioned before.

Moreover, the graph also depicted that 50 g/L glucose and galactose gave the highest MC concentration because the optimum condition of media containing the suitable concentration of carbon source, enhanced the MC concentration. Furthermore, the gradual increment trend of MC concentration could be due to the stable and efficient supply of oxygen throughout the experiment. A study by Pourramezan *et al.* (2009) demonstrated that the higher the concentration of sugar concentration used, the higher the sugar consumption for bacteria growth, the higher the cell concentration, thus, the higher the MC production. The result of this study was agreed by the research by Adnan (2015). Hence, 50 g/L of glucose and galactose is the optimum concentration for cell metabolism of bacteria and absorption of other nutrients to produce MC. However, it showed that 70 g/L glucose and galactose gave the lowest MC concentration although the concentration of media increased. This is because the excess carbon source provided would disrupted the cell metabolism of bacteria and affected the osmotic pressure of bacteria

cells. Hence, the enzyme inside cells could not utilize the presence of carbon source and other nutrients efficiently.

Besides, the lowest MC production could be due to high concentration of glucose are utilized, the higher the formation of gluconic acid as by product, which cause pH of media decrease significantly and directly affect the viability of bacteria inside the media (Chawla *et al.,* 2009). This caused the MC production become lower than expected result. Nevertheless, researchers strongly recommend that lignosulphonate can be added into the culture media in order to reduce the formation of gluconic acid (Chawla *et al.,* 2009). According to a study by Masaoka *et al.* (1993), it was claimed that MC yields decreased when beyond 40 g/L of initial concentration of glucose was used, however, most of other researchers had tried higher concentrations of sugar.

Effects of varying the glucose and galactose combination concentration on MC volumetric productivity

Based on Table 2, 10, 30 and 50 g/L of glucose and galactose combination, the volumetric productivity of MC is directly proportional to the MC concentration, which showed 0.008 g/L/day of volumetric productivity, while lowest value for 70 g/L of sugar (0.004 ± 0.003 g/L/day).

CONCLUSION

The best combination of dual-saccharides to enhance MC production by *P. aeruginosa* were determined and investigated. Among the various combination of dual-saccharides, the glucose and galactose media gave the highest yields of MC of 1.23 ± 0.15 g/L. and 0.007 ± 0.001 g/L/day of volumetric productivity. For the optimization study, it can be concluded that 50 g/L of glucose + galactose was the best concentration for MC production as it produced high MC yield. Increasing the concentration of glucose + galactose beyond 50 g/L decreased MC production and also MC volumetric productivity.

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